Heliyon 9 (2023) e21800

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Network pharmacology and *in vivo* studies reveal the neuroprotective effects of paeoniflorin on Alzheimer's disease

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ARTICLE INFO

Keywords:
Paeoniflorin
Alzheimer's disease
Network pharmacology
Oxidative stress
Neuroinflammation

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease that has still not been effectively treated. Paeoniflorin is a traditional Chinese medicine with potential neuroprotective effects against brain injury; however, the beneficial effects and mechanisms of action in AD have not been clarified. We aimed to explore the mechanisms of action of paeoniflorin in AD using network pharmacology and experimental validation. Network pharmacology analysis revealed 30 candidate targets through the intersection of the targets of paeoniflorin and related genes in AD, which were mainly enriched in oxidative stress and inflammation. Moreover, key targets of paeoniflorin against AD, namely Nrf2 (encoded by NFE2L2) and TLR4, were screened and found to be closely related to oxidative stress and inflammation. Subsequent *in vivo* experiments revealed that paeoniflorin treatment improved the cognition of APP/PS1 mice by ameliorating oxidative stress and neuroinflammation, which were associated with the upregulation of Nrf2 and HO1, and the downregulation of TLR4. Collectively, the present study demonstrates that paeoniflorin alleviates cognitive impairment in AD by regulating oxidative stress and neuroinflammation, and that Nrf2, HO1, and TLR4 could be key targets.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by cognitive dysfunction and memory loss [1]. As the most common type of dementia in individuals older than 60 years, AD affects more than 50 million people worldwide and is expected to surge to 152 million by 2050 [2]. The typical pathological characteristics of AD include amyloid-β (Aβ) plaques, oxidative stress, and neuroinflammation [3,4]. Commonly used anti-AD drugs are donepezil, rivastigmine, and memantine, which only treat symptoms but do not impede disease progression and are accompanied by many side effects [5,6]. Therefore, safer and more effective drugs are required for treating AD.

Many studies have reported the use of traditional Chinese medicine in the treatment of AD [7]. Paeoniflorin, a highly water-soluble single-terpenoid glycoside, is the main component of the Chinese herb *Paeonia lactiflora Pall*. Growing evidence has demonstrated various pharmacological activities of paeoniflorin such as anti-inflammatory, anti-oxidative, anti-apoptotic, and immune regulation [8–10]. It also plays a neuroprotective role in the central nervous system. Wu et al. demonstrated that oral treatment with paeoniflorin

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https://doi.org/10.1016/j.heliyon.2023.e21800
Received 5 April 2023; Received in revised form 19 September 2023; Accepted 28 October 2023
Available online 31 October 2023

in a cerebral ischemic rat model ameliorated neurological deficits, reduced the infarction area, and relieved cerebral edema through its antioxidant effects [11]. In addition, previous studies have demonstrated that paeoniflorin alleviates cognitive impairments in animal models of AD [12,13]. However, molecular mechanisms underlying the neuroprotective effects of paeoniflorin in AD remain unclear.

As an emerging approach for drug discovery and development, network pharmacology has been applied to comprehensively clarify the pharmacological effects of traditional Chinese medicines on various diseases [14,15]. Network pharmacology is a systematic approach that integrates traditional pharmacology, bioinformatics, chemoinformatics, and systems biology, making it possible to establish drug-target-disease interaction networks and shedding light on the development of novel drugs for disease treatment [16]. Network pharmacology has been extensively used to elucidate multi-target effects in central nervous system diseases such as AD, vascular cognitive impairment [17], and ischemic stroke [18].

The present study aimed to explore the therapeutic targets of paeoniflorin and uncover its anti-AD mechanisms using network pharmacology. We screened 30 paeoniflorin targets in AD. The anti-AD effects of paeoniflorin, as suggested by the network analysis, were further verified by molecular docking and biological experiments. Our study provides a systematic pharmacological basis for the treatment of AD using paeoniflorin.

2. Materials and methods

2.1. Network pharmacology analysis

2.1.1. Identification of potential targets of paeoniflorin

Structural information on paeoniflorin was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The predicted targets of paeoniflorin were searched and collected from the following databases: Comparative Toxicogenomics Database (CTD) (HTTPS://ctdbase.org) and STITCH (http://stitch.embl.de/). Duplicate targets were eliminated, and related targets were retained.

2.1.2. Identification of potential targets of AD

DisGeNET (https://www. disgenet. org/) and Genecards (https://www. genecards. org/) databases were used to screen for potential AD targets. Duplicate targets were eliminated, and related targets were retained.

2.1.3. Construction and analysis of the protein-protein interaction (PPI) network

Overlapping target genes were obtained using Veeny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). Common target genes were submitted to the STRING database (version 11.0, https://www.string-db.org/) to construct a PPI network. To ensure the reliability of the data, a confidence score $\geq$0.4 was set to acquire targets with"*Homo sapiens*." PPI results were imported into the Cytoscape software (version 3.4.0, http://chianti.ucsd.edu/cytoscape-3.4.0/) for network production and analysis.

2.1.4. Go and KEGG enrichment analysis

Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analyses were performed using ClusterProfiler (version:3.8.1, http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html).

2.1.5. Molecular docking

AutoDock Vina software (http://autodock.scripps.edu/) was used to analyze the molecular binding affinity of molecular docking between paeoniflorin and the core targets of AD. The three-dimensional structure of paeoniflorin was retrieved from GenBank (https://www.genecards.org/) and the crystal structures of the core target proteins, including NFE2L2/nuclear factor erythrocyte 2-related factor 2 (Nrf2) and toll like receptor 4 (TLR4), were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/). PyMOL (https://pymol.org/2/) and AutoDock were used for the structural modification of these proteins, including the removal of water molecules and heteroatoms and the addition of charges and hydrogen atoms, and the proteins were subsequently stored in the PDBQT format for binding. Subsequently, a docking simulation was performed using AutoDock Vina, and the docking morphology was visualized using PyMOL.

2.2. In vivo experimental study

2.2.1. Animals and treatment

Male APPswe/PS1dE9 (APP/PS1) double-transgenic mice with a C57BL/6J background were obtained from the Jackson Laboratory (No. 005864; Bar Harbor, ME, USA). Mice were housed in a pathogen-free environment maintained at a controlled temperature of 23 $\pm$ 2 °C, 12 h of light alternating with darkness, relative humidity of 60 $\pm$ 5 %, and with free access to food and water. The experiments were performed in compliance with The Guidelines for Animal Care and Use of China, and all the animal experimental procedures were approved by the Experimental Animal Center of the Anhui University of Science and Technology.

Eight-month-old male APP/PS1 and WT mice were divided into three groups: wild type mice treated with the vehicle (WT group), APP/PS1 mice treated with the vehicle (APP/PS1 group), and APP/PS1 mice treated with paeoniflorin (Pae group). Paeoniflorin (purity, $\geq$98 %) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and its chemical structure is shown in Fig. 2A. Paeoniflorin was administered intraperitoneally at 5 mg/kg/day for 4 weeks, which was selected based on previous studies [13].

2.2.2. Y maze

The Y-maze was used to assess working memory. The Y-maze apparatus consisted of three symmetrical arms placed at a120-degree angle (15 cm high, 30 cm long, and 8 cm wide). The mice were placed in the center and allowed to explore freely through the Y-maze for 5 min. The number of arm entries was video-recorded and automatically quantified using EthoVision XT software (Noldus). Correct alternations were defined as consecutive entries into three different arms. The maximum alternation was defined as the number of possible alternations counted as the total number of arms entered minus two. Alternation (%) was calculated as the number of correct alterations/maximum alternations.

2.2.3. Morris water maze

The Morris water maze test was used to evaluate spatial learning and memory in mice, and was performed as described previously [19]. The apparatus was 120 cm in diameter and 50 cm high. A circular platform was submerged in the first quadrant 1 cm below the water surface. The procedure included navigation and spatial probe tests. During the navigation test, the mice were trained in four trials per day to escape onto the platform within 60 s for 5 consecutive days. Otherwise, the mice were directed to the platform and kept there for 10 s. During the probe trial, the platform was removed and the mice were allowed to swim freely for 60 s. The escape latency, number of platform crossings, and average swimming speed were recorded and automatically quantified using the EthoVision XT software (Noldus).

2.2.4. Thioflavin S fluorescence staining

Thioflavin S staining was performed as previously described [20]. Briefly, the brain sections were deparaffinized in xylene and hydrated using a series of graded ethanol solutions (100 %, 90 %, 80 %, and 70 %). Thereafter, the sections were incubated in 1 % Thioflavin S staining solution for 5 min, differentiated in 70 % ethanol for 1 min, and mounted. All the sections were imaged using an Olympus microscope.

2.2.5. Measurement of superoxide dismutase (SOD) activities and malondialdehyde (MDA) levels

The hemispheres of mice were homogenized on ice and centrifuged to collect the supernatants. Supernatants were used to detect the SOD activity and MDA levels using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All the procedures were performed according to manufacturer's instructions.

2.2.6. Enzyme-linked immunosorbent assay (ELISA) analysis

The interleukin (IL)-1β, IL-6, TNFα, $A\beta_{1\text{-}40}$, and $A\beta_{1\text{-}42}$ levels were measured using ELISA. Briefly, the brain samples were thawed on ice, homogenized, and centrifuged at 12,000×*g* at 4 °C for 30 min. The supernatants were collected and protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime, P0010, Shanghai, China). The IL-1β, IL-6, TNFα, $A\beta_{1\text{-}40}$, and $A\beta_{1\text{-}42}$ levels were then quantified using ELISA kits according to the manufacturer's protocols (Multisciences, Hangzhou, China).

2.2.7. Immunofluorescence staining

Brain tissues were fixed in 4 % paraformaldehyde, embedded in paraffin, and cut into 4- μm thick sections. Sections were deparaffinized in xylene, hydrated with a series of graded ethanol concentrations (100 %, 90 %, 80 %, and 70 %), and heated in a microwave oven for antigen retrieval. Subsequently, sections were blocked with 5 % BSA, followed by overnight incubation with 8-hydroxy-2′-deoxyguanosine (8-OHdG) (1:200, Bioss, Beijing, China) and Iba-1 (1:100, Servicebio, Wuhan, China) primary antibodies at 4 °C. Thereafter, the sections were washed thrice with PBS and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) secondary anti-rabbit antibody (1:1000, Servicebio, Wuhan, China). After triple washing in PBS, the sections were counterstained with DAPI to dye the nuclei. Sections were visualized and analyzed using fluorescence microscopy.

2.2.8. Western blotting

Western blotting was performed as previously described [21]. Briefly, protein concentrations in the brain were measured using a BCA Protein Assay Kit (Beyotime, P0010, Shanghai, China). Equivalent amounts of protein were then sequentially loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membranes, blocked with 5 % BSA, and incubated with primary antibodies for Nrf2 (1:1000, Proteintech, IL, USA), HO1 (1:1000, Abclonal, Wuhan, China), toll like receptor 4 (TLR4) (1:200, Santa Cruz, USA), and β-actin (1:1000, Proteintech, IL, USA) overnight at 4 °C. After washing with TBST for 3 times, the membranes were incubated with HRP)conjugated IgG secondary antibody (1:1000, Servicebio, Wuhan, China) for 1 h at room temperature. Immunoblots were visualized using an enhanced chemiluminescence kit (Invitrogen, Carlsbad, CA, USA) and the densities were quantified using ImageJ software.

2.2.9. Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM) and statistical analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., CA, USA). Differences between the groups were determined using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. For experiments that included only two groups, a *t*-test was used. For the navigation test of the Morris water maze, escape latency was analyzed using two-way repeated-measures ANOVA, followed by Tukey's post-hoc test. $P < 0.05$. significant.

3. Results

3.1. Network pharmacology analysis of paeoniflorin against AD

Network pharmacology analysis was performed to explore the potential targets of paeoniflorin in AD (Fig. 1). A total of 34 paeoniflorin targets were identified using the CTD and STITCH databases and 2725 AD related genes were screened from the DisGeNET and GeneCards databases. According to the Venn diagram shown in Fig. 2B and 30 common targets of paeoniflorin and AD were identified through the intersection of the targets of paeoniflorin and related genes in AD. Thirty intersecting targets were imported into the STRING database to construct a PPI network (Fig. 2C and D). To better understand the biological functions and signaling pathways involving intersecting targets between paeoniflorin and AD, the common targets of paeoniflorin and AD were enriched by GO and KEGG analyses using the R package. The biological processes of GO function analysis revealed that the targets of paeoniflorin against AD were mainly enriched in oxidative stress-, inflammation-, and apoptosis-related terms, including response to oxidative stress, regulation of reactive oxygen species (ROS) metabolic processes, negative regulation of immune system processes, intrinsic apoptotic signaling pathways, and neuronal death (Fig. 2E). The top pathways significantly enriched in the KEGG analysis mainly included AD, TNF signaling pathway, toll-like receptor signaling pathway, and apoptosis (Fig. 2F). The abovementioned GO and KEGG analyses suggest that oxidative stress and inflammation could be potential mechanisms involved in the treatment of AD with paeoniflorin.

3.2. Paeoniflorin ameliorates cognitive impairment in APP/PS1 mice

The Y- and Morris water maze tests were performed to assess the cognitive abilities of APP/PS1 mice after 4 weeks of paeoniflorin treatment (Fig. 3A). In the Y-maze test, we observed that compared with the WT control, APP/PS1 mice showed lower alternation behavior. After paeoniflorin treatment, APP/PS1 mice showed significantly improved alternation behaviors (Fig. 3B). Subsequently, Morris water maze test was performed to investigate whether paeoniflorin improves spatial learning and memory in APP/PS1 mice.

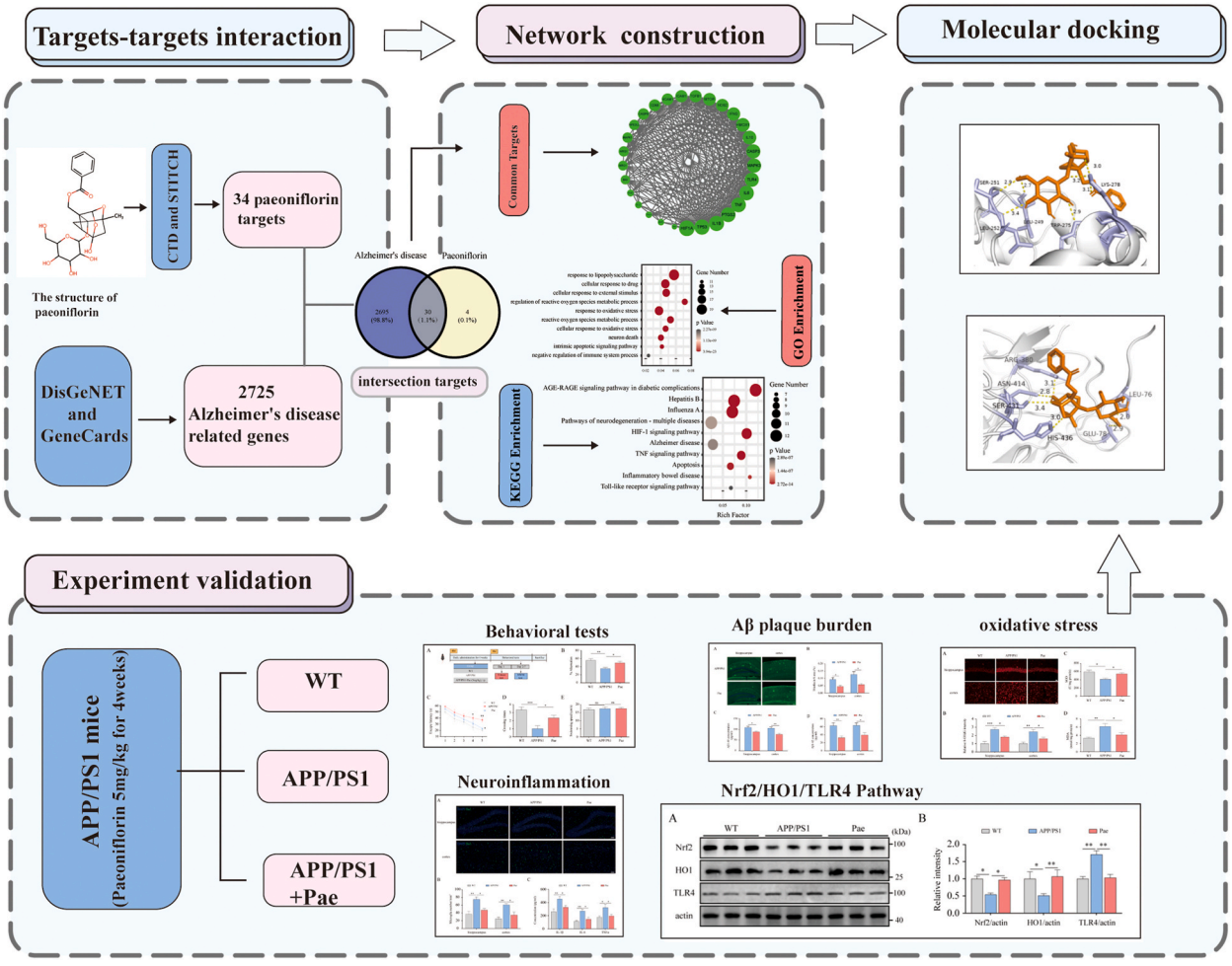

Fig. 1. Flowchart of network pharmacology to investigate the potential mechanisms of paeoniflorin against Alzheimer's disease.

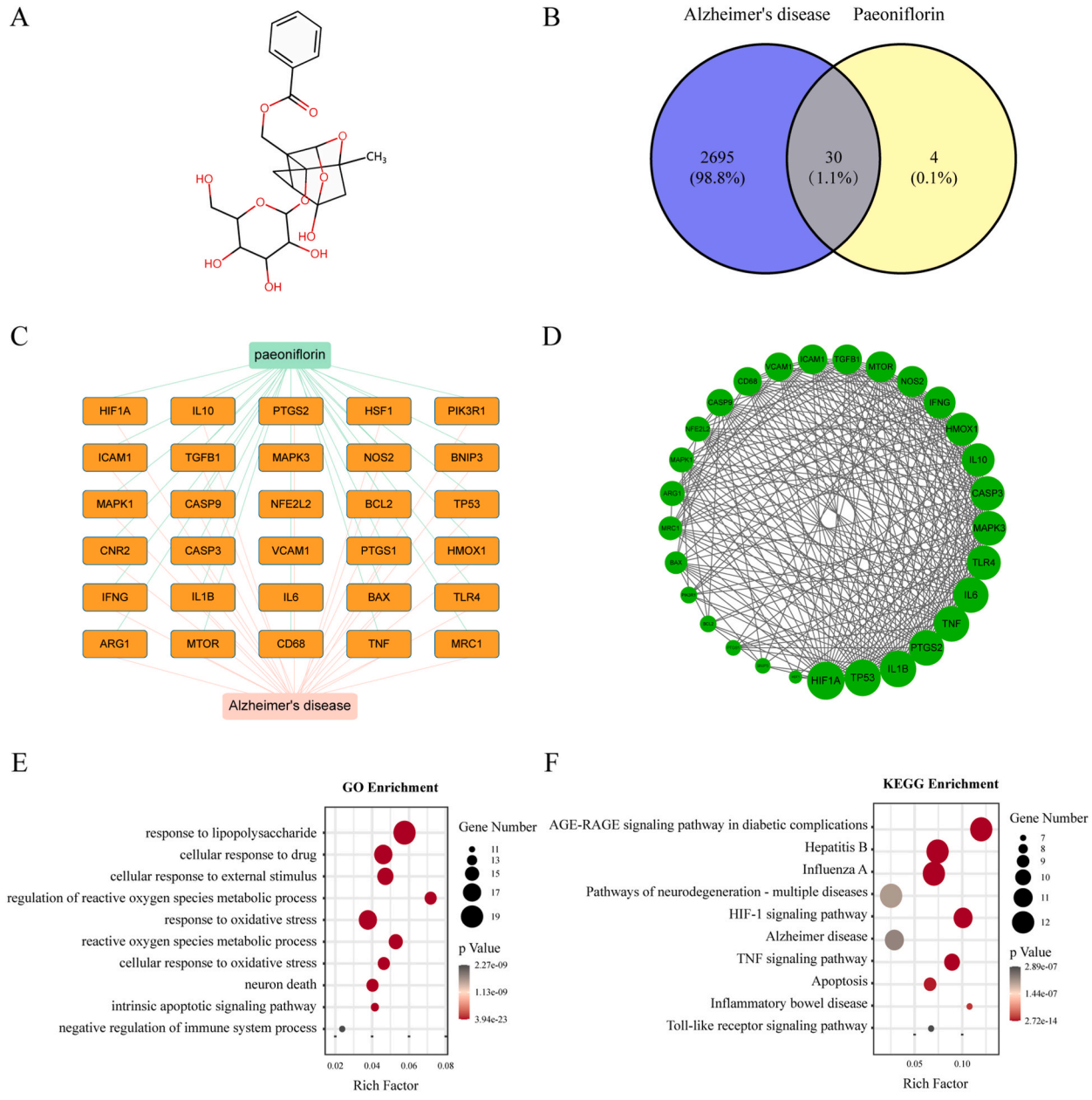

Fig. 2. Network pharmacology analysis of paeoniflorin against Alzheimer's disease (AD). (A) Chemical structure of paeoniflorin. (B) Venn diagram of potential targets. (C, D) The Protein-Protein interaction network map of 30 target genes. (E) Analysis of Gene Ontology biological function enrichment for paeoniflorin-treated AD. (F) Analysis of Kyoto Encyclopedia of Genes and Genomes pathway enrichment for paeoniflorin-treated AD.

The Morris Water Maze test showed a gradual decrease in escape latency in all the groups tested (Fig. 3C). However, APP/PS1 mice exhibited a significantly higher escape latency on days 4 and 5 than the WT control, whereas the escape latency of the paeoniflorin-treated APP/PS1 mice was markedly reduced on day 5 compared to that of the APP/PS1 mice (Fig. 3C). During the probe trial, APP/PS1 mice showed fewer crossing times in the former platform position and spent a significantly shorter time in the target quadrant than the WT control. However, the number of platform crossings and the percentage of time spent in the target quadrant in paeoniflorin-treated APP/PS1 mice were remarkably increased compared with those in APP/PS1 mice (Fig. 3D and E). No statistically significant differences were observed in the swimming speed among the three groups (Fig. 3F). Taken together, these results revealed that cognitive impairment in APP/PS1 mice could be improved by paeoniflorin treatment.

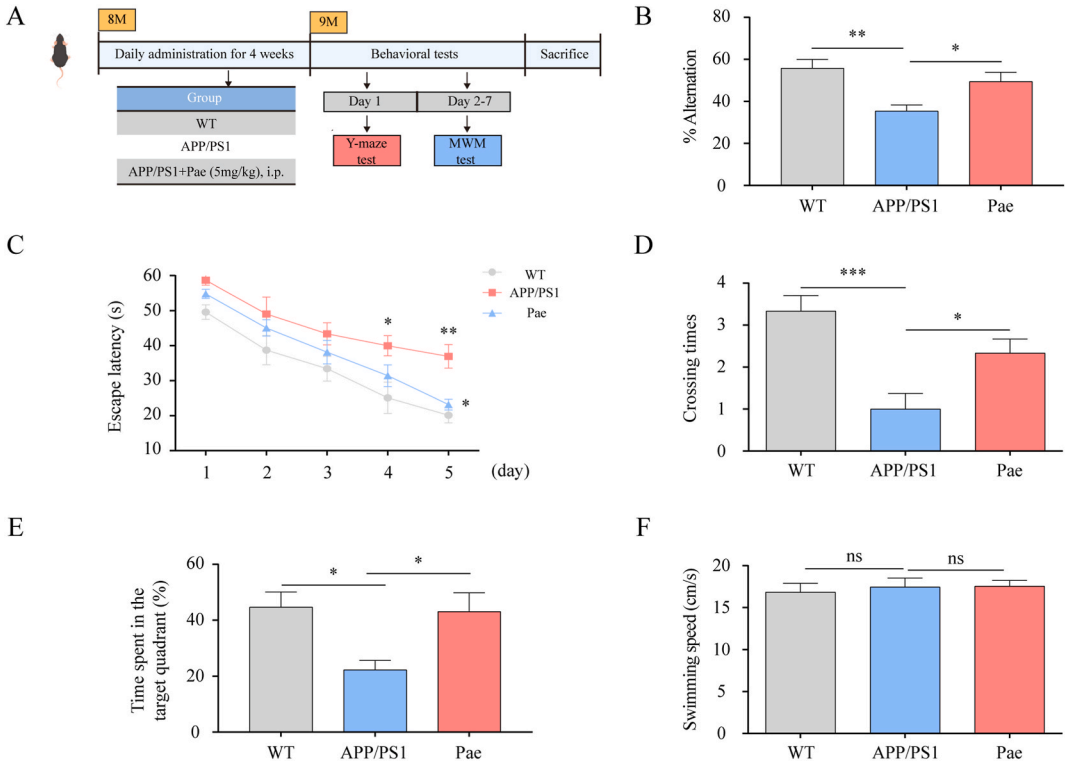

Fig. 3. Paeoniflorin ameliorates cognitive impairment in APP/PS1 mice. (A) Schematic diagram of the experimental procedure. (B) The percentage of spontaneous alternation in Y maze analysis. n = 9 mice/group. (C) The escape latency of mice in the training trials of the hidden platform task. n = 9 mice/group. (D) Frequency of platform crossing in the probe trial. n = 9 mice/group. (E) Percentage of time spent in the target quadrant in the probe trial. n = 9 mice/group. (F) Swimming speed in the probe trial. n = 9 mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.3. Paeoniflorin alleviates plaque burden and Aβ levels in the APP/PS1 mice

Since the accumulation of amyloid plaques is one of the key pathological hallmarks of AD, Thioflavin-S staining and ELISA were performed to investigate whether paeoniflorin treatment affected Aβ deposition. As expected, a significant decrease in the amyloid plaque area was observed in the hippocampus and cortex of paeoniflorin-treated APP/PS1 mice (Fig. 4A and B). Consistent with the Thioflavin-S staining, paeoniflorin treatment lowered both the $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the hippocampus and cortex of APP/PS1 mice (Fig. 4C and D). The above data suggested that paeoniflorin treatment alleviates Aβ plaque burden in APP/PS1 mice.

3.4. Paeoniflorin ameliorates oxidative stress in APP/PS1 mice

Excessive oxidative stress has also been implicated in the progression of AD [22]. Moreover, GO functional analysis revealed that the targets of paeoniflorin in AD were mainly enriched in oxidative stress (Fig. 2E). Therefore, the levels of oxidative stress markers (8-OHdG, SOD, and MDA) were assessed. As shown in Fig. 5A and B, the intensity of 8-OHdG in the hippocampus and cortex was higher in APP/PS1 mice than in WT controls. In the paeoniflorin-treated group, the elevated 8-OHdG levels evidently decreased. In addition, we observed significantly lower SOD activity and higher MDA levels in APP/PS1 mice, whereas paeoniflorin treatment prominently reversed these changes in the brains of APP/PS1 mice (Fig. 5C and D). Collectively, these results indicated that paeoniflorin ameliorated oxidative stress in APP/PS1 mice.

3.5. Paeoniflorin suppresses microglial activation and proinflammatory cytokine levels in APP/PS1 mice

Increasing evidence indicates that oxidative stress is closely related to neuroinflammation in the brain of AD mice [23]. Sustained overactivation of the microglia accelerates the progression of AD by releasing proinflammatory cytokines, leading to neuronal damage and cognitive impairment [24]. Consistent with previous results, we found that the number of microglia increased in both the hippocampus and cortex of APP/PS1 mice compared to that of the WT control, whereas paeoniflorin administration markedly reduced the number of microglia in APP/PS1 mice (Fig. 6A and B). In addition, ELISA results showed that the IL-1β, IL-6, and TNF-α expression was significantly higher in the APP/PS1 mouse brain than in the WT control. However, the expression of these proinflammatory cytokines in APP/PS1 mice decreased after paeoniflorin treatment (Fig. 6C). These results indicate that paeoniflorin effectively suppressed microglial activation and proinflammatory cytokine levels in APP/PS1 mice.

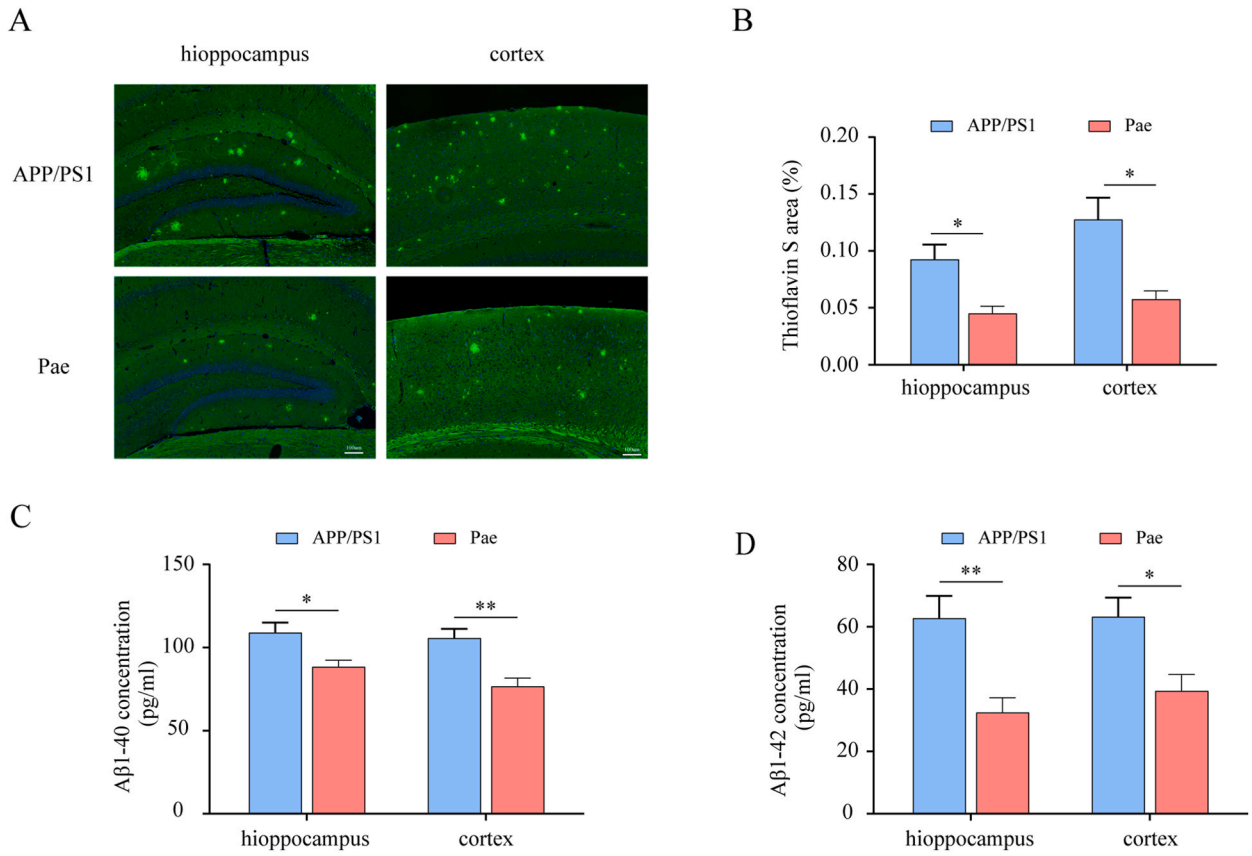

Fig. 4. Paeoniflorin alleviates plaque burden and Aβ levels in APP/PS1 mice. (A) Representative images of Aβ staining with Thioflavin S in the hippocampus and cortex of paeoniflorin-treated APP/PS1 mice. Scale bar = 100 μm. (B) Quantification of Thioflavin S percent area in the hippocampus and cortex of paeoniflorin-treated APP/PS1 mice. n = 4 mice/group. (C) The $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were measured in the hippocampus and cortex from APP/PS1 and paeoniflorin-treated APP/PS1 mice using ELISA. n = 6 mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.6. Effect of paeoniflorin on the Nrf2/HO1/TLR4 pathway in APP/PS1 mice

Studies have shown that Nrf2 is a key regulator of oxidative stress and inflammatory response [25]. Moreover, network pharmacology analysis revealed that Nrf2 and TLR4 are key paeoniflorin-mediated proteins against AD (Fig. 2C and D). To elucidate the mechanisms underlying the neuroprotective effects of paeoniflorin, the expression of the Nrf2/HO1/TLR4 signaling pathway was validated by western blotting. The expression of Nrf2 and its downstream effector HO1 was significantly decreased in APP/PS1 mice, whereas paeoniflorin treatment dramatically increased Nrf2 and HO1 expression (Fig. 7A and B). In contrast, the expression of TLR4 was markedly increased compared to that in the WT control, whereas APP/PS1 mice showed a remarkable decrease in the expression of TLR4 following paeoniflorin treatment (Fig. 7A and B). Furthermore, molecular docking was used to verify the possibility of paeoniflorin binding to Nrf2 and TLR4 via AutoDock Vina. Molecular docking revealed that the affinity of paeoniflorin and Nrf2 was −7.6 kcal/mol and the affinity of paeoniflorin and TLR4 was −6.7 kcal/mol, suggesting that paeoniflorin was stably combined with Nrf2 and TLR4 (Fig. 7C and D). Overall, our findings indicated that paeoniflorin may play antioxidant and anti-inflammatory roles by altering the expression of key proteins in AD.

4. Discussion

AD is a multifactorial neurodegenerative disease characterized by progressive cognitive decline associated with Aβ plaques deposition, neuroinflammation, and oxidative stress [26]. Given its complex etiology and pathogenesis, curative treatments are still lacking. Currently, the drugs used to treat AD are mainly single-target drugs that only delay the symptoms of AD to a certain extent and have a number of side effects [27,28]. Therefore, it is imperative to explore multi-target anti-AD drugs that have important practical significance and broad application prospects. Recently, there has been increasing interest in the use of traditional Chinese medicine in the treatment of AD owing to its advantages of multiple targets and fewer side effects. Various traditional Chinese medicines including berberine, forsythoside, Tribulus terrestris, and resveratrol have been used in the prevention and treatment of AD [29–32]. For example, studies have demonstrated that berberine may exhibit a protective effect by preventing the disaggregation of Aβ plaques and

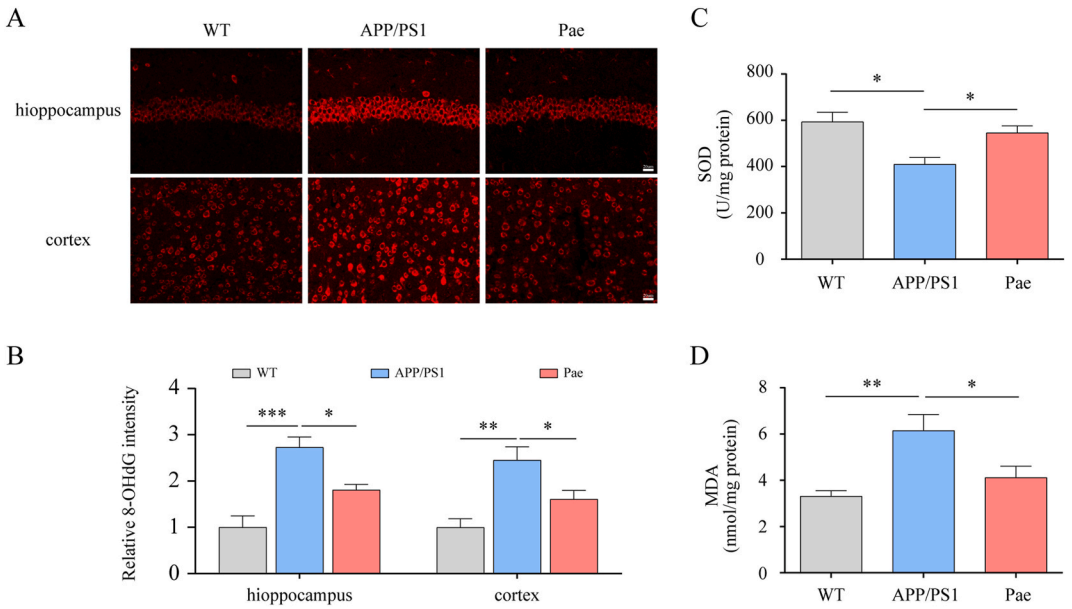

Fig. 5. Paeoniflorin ameliorates oxidative stress in APP/PS1 mice. (A) Representative immunofluorescent staining of 8-hydroxydeoxyguanosine (8-OHdG)-positive cells in the hippocampus and cortex of mice. Scale bar = 20 μm. (B) Quantitative analysis of relative 8-OHdG intensity in the hippocampus and cortex. n = 3 mice/group. (C) The superoxide dismutase activities in the brain of APP/PS1 mice after treatment with paeoniflorin were measured. n = 4 mice/group. (D) The malondialdehyde levels in the brain of APP/PS1 mice after treatment with paeoniflorin were measured. n = 4 mice/group.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

limiting neuroinflammation and oxidative stress in AD [33].

Paeoniflorin, the primary active constituent of *Paeonia lactiflora Pall*, has been found to have preventive and therapeutic properties against various nervous system disorders [10,11]. Luo et al. reported that paeoniflorin administration ameliorated cognitive deficits in a rat model of vascular cognitive dysfunction induced by common carotid artery clipping [34]. Similarly, another study by Kong et al. has demonstrated that paeoniflorin supplementation improves learning and memory abilities of transgenic mice with five familial Alzheimer's disease (5 × FAD) mutations by alleviating Aβ plaque burden [13]. Consistent with previous reports, we found that paeoniflorin treatment evidently improved learning and memory capacity and alleviated Aβ plaque burden of APP/PS1 mice, suggesting that dietary supplementation with paeoniflorin may exert a variety of beneficial effects on AD. However, the exact mechanism underlying the neuroprotective effects of paeoniflorin in AD remains unclear.

Network pharmacology, based on the biology, pharmacology, and computational algorithms, was used to predict potential targets and mechanisms of traditional Chinese medicine from a systematic perspective [35]. Network pharmacology is widely used to identify anti-AD herbal medicines and their molecular targets. For example, the molecular targets of cordycepin and palmatine in AD were previously identified and validated using a network pharmacology approach [36,37]. Therefore, in the present study, network pharmacology was used to explore the therapeutic targets and related mechanisms of action of paeoniflorin in AD. Network pharmacology analysis revealed that 34 paeoniflorin and 2725 AD-related targets were screened, of which 30 targets were shared between paeoniflorin- and AD-related targets, suggesting that paeoniflorin may improve AD through these targets. Thirty intersecting targets were further enriched using GO and KEGG analyses. The results revealed that the targets of paeoniflorin against AD were mainly enriched in oxidative stress, toll-like receptor signaling pathway, TNF signaling pathway, and AD, suggesting that oxidative stress and inflammation might be potential mechanisms involved in the treatment of paeoniflorin in AD.

Accumulating evidence suggests that oxidative stress is involved in AD progression [38,39]. Previous studies have shown extensive oxidative damage in postmortem AD brain samples compared to age-matched controls [40,41]. In addition, increased oxidative stress in AD brains is evidenced by increased levels of oxidative damage markers of mitochondrial DNA, such as 8-OHdG [42]. Lipid peroxidation products, such as MDA and 4-hydroxynonenal are also elevated in the AD brain [43,44]. Moreover, a reduction in the activity of antioxidant enzymes, including SOD, catalase, and glutathione peroxidase (GSH-Px), has been observed in the brains of patients with neuropathologically confirmed AD [45]. Oxidative stress has been extensively investigated in rodent models of AD. For instance, the levels of oxidative stress, indicated by the production of ROS, 4-hydroxy-nonenal, and 8-OHdG, were significantly increased in the hippocampus of $A\beta_{1\text{-}42}$-induced AD mice [46]. APP/PS1 transgenic mice, a typical animal model of AD, exhibited increased MDA levels and decreased SOD and GSH-Px activities compared to age-matched controls [47]. In line with previous studies, we observed increased oxidative stress in APP/PS1 mice compared to WT controls, as evidenced by higher levels of 8-OHdG and MDA and lower activity of SOD. However, paeoniflorin treatment significantly reversed these changes in APP/PS1 mice, suggesting that paeoniflorin inhibits oxidative stress in AD. A previous study showed that paeoniflorin attenuates oxidative stress by upregulating Nrf2/HO1 in a rat model of subarachnoid hemorrhage [10]. In addition, Wang et al. revealed that 6′-*O*-galloylpaeoniflorin, a

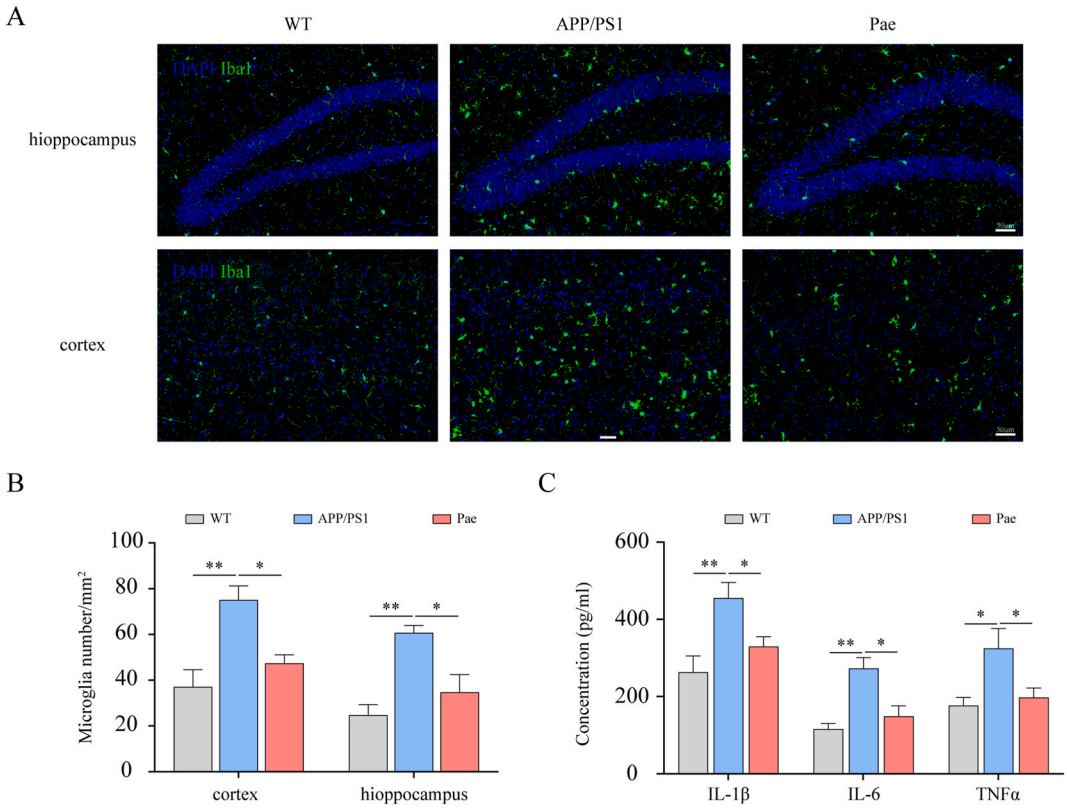

Fig. 6. Paeoniflorin suppresses microglial activation and proinflammatory cytokine levels in APP/PS1 mice. (A) Representative immunofluorescent staining of Iba1-positive microglia in the hippocampus and cortex of mice. Scale bar = 50 μm. (B) Quantification of Iba1-staining positive cells within the hippocampus and cortex. n = 3 mice/group. (C) Enzyme-linked immunosorbent assay was performed to detect the protein levels of IL-1β, IL-6, and TNFα in the brain of APP/PS1 mice after treatment with paeoniflorin. n = 3 mice/group.*$P < 0.05$; **$P < 0.01$.

galloylated derivative of paeoniflorin, attenuates cerebral ischemia/reperfusion-induced oxidative stress via Nrf2 activation [48]. As a redox-sensitive transcription factor and key regulator of cellular responses to oxidative stress, Nrf2 maintains redox homeostasis by regulating downstream proteins and enzymes, such as HO1 and SOD. However, nuclear Nrf2 levels have been reported to be diminished in the CA1 region of patients with AD [49,50]. Moreover, boosting the activity of the Nrf2-ARE pathway by overexpressing Nrf2 protects against Aβ toxicity in APP/PS1 transgenic mice [51]. In the present study, we found that Nrf2 and HO1 was reduced in APP/PS mice, whereas paeoniflorin treatment significantly enhanced Nrf2 and HO1 expression. In general, our findings demonstrated that paeoniflorin exerts an anti-oxidative effect by upregulating Nrf2/HO1 in APP/PS1 mice.

Elevated levels of inflammatory markers in individuals with AD and the identification of AD risk genes related to the innate immune function indicate that neuroinflammation plays an important role in the development of AD [52], which is characterized by the chronic and persistent activation of the microglia triggered by misfolded or aggregated proteins, such as Aβ, and the subsequent release of pro-inflammatory cytokines [53]. TLR4, which is widely expressed on the microglial membrane of the central nervous system, is involved in recognizing Aβ and regulating neuroinflammation [54]. Upon binding with Aβ, TLR4 interacts with the NF-κB complex, leading to the production of pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα [55]. Cheng et al. demonstrated that paeoniflorin inhibited TLR4/NF-κB/NLRP3 signaling, reduced microglial activation, and decreased proinflammatory cytokine levels in the hippocampus of LPS-induced mice [56]. Moreover, a recent study has demonstrated that paeoniflorin supplementation alleviated neonatal hypoxic brain injury by preventing microglial activation and inflammatory cytokines production via TLR4/NF-κB signaling [57]. Our study demonstrated that APP/PS1 mice exhibited increased microglial activation, proinflammatory cytokine levels, and TLR4 expression, which were effectively improved by paeoniflorin administration. Therefore, our findings suggest that paeoniflorin may exert an anti-inflammatory effect on AD by regulating TLR4 signaling.

In conclusion, we comprehensively and systematically analyzed the targets and mechanisms of paeoniflorin in AD by combining a network pharmacology approach and experimental validation in the present study. These results revealed that paeoniflorin treatment improved cognitive impairment in APP/PS1 mice by ameliorating oxidative stress and neuroinflammation via the Nrf2/HO1/TLR4 pathway. Collectively, these data suggest that paeoniflorin is a promising candidate drug for AD treatment. Moreover, the role of the Nrf2/HO1/TLR4 pathway in paeoniflorin for AD treatment needs to be further validated using methods, such as gene editing in mice, because molecular docking is not sufficient for the interaction.

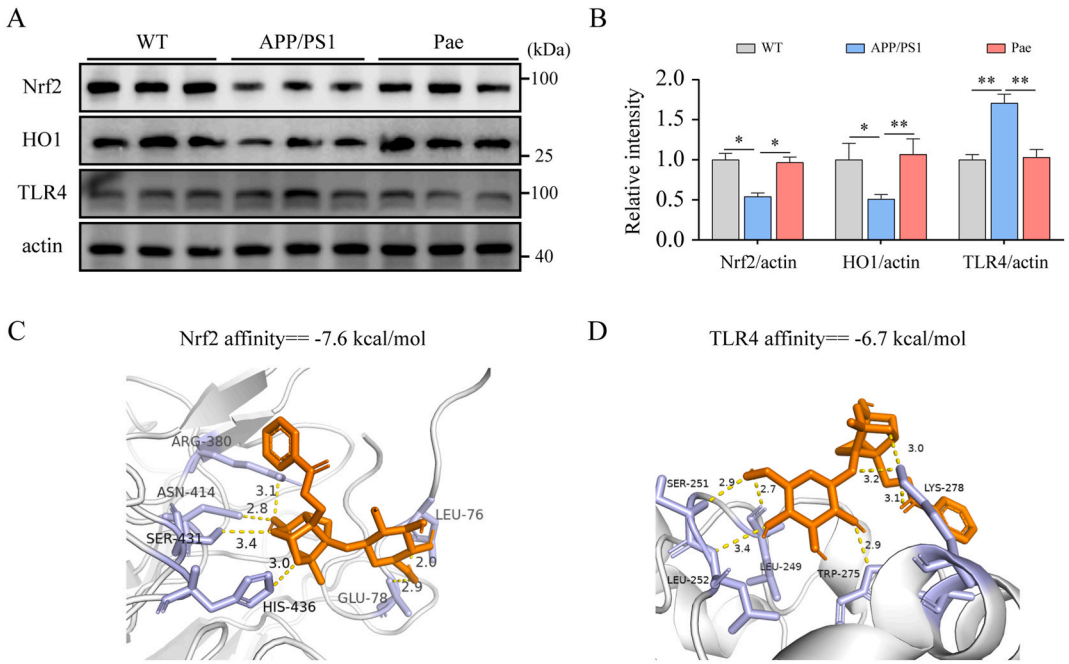

Fig. 7. Effect of paeoniflorin on the nuclear factor erythrocyte 2-related factor 2 (Nrf2)/Heme oxygenase 1 (HO1)/toll like receptor 4 (TLR4) pathway in APP/PS1 mice. (A) Representative immunoblotting bands of Nrf2, HO1, and TLR4 in the brain of APP/PS1 mice following paeoniflorin treatment. n = 3 mice/group. (B) Quantitative analysis of Nrf2/actin, HO1/actin, and TLR4/actin. n = 3 mice/group. (C) Paeoniflorin docking with Nrf2. (D) Paeoniflorin docking with TLR4. $^{*}P < 0.05$; $^{**}P < 0.01$.

Author contribution statement

Mengyuan Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Haoran Zheng: Performed the experiments; Analyzed and interpreted the data. Jiale He: Analyzed and interpreted the data. Mei Zhang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This study was supported by a research grant from the Science and Technology Project of Anhui Province (Grant No. 12010402124).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21800.

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